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Isolation and concentration of *Salmonellae* with an immunoaffinity column☆

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Abstract

A method for rapidly and selectively isolating *Salmonellae* from buffer solutions and concentrating the bacteria by a factor of ~ 500 was developed. Anti-*Salmonellae* antibody was covalently linked to 40 μm polyacrylamide beads to prepare a solid phase with affinity for the bacteria. The beads were packed into 1-mm diameter glass tubes to form a column 20 μl in volume. Buffer containing *Salmonellae* at concentrations ranging from 10^2 to 10^6/ml was pumped through the column to trap and concentrate the bacteria. At a flow rate of 50 μl/min, more than 95% of the bacteria introduced to the column were captured, while at 800 μl/min capture dropped to 32%. Specificity was high, with no detectable capture of *Escherichia coli* at a concentration of 10^5/ml. Capture of more than 90% of *Salmonellae* in a 5-ml sample was achieved in 40 min by re-circulating the sample through the column at a flow rate of 500 μl/.
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Keywords: Immunoaffinity; *Salmonellae*; Bacteria; Detection

1. Introduction

Illness caused by foodborne pathogenic bacteria is a significant public health problem, and efforts to address this problem are dependent on rapid, sensitive methods to detect pathogens in foodstuffs. The infectious dose for many foodborne pathogenic bacteria is a few cells, equivalent to less than a picogram dry weight. Selective detection of this small mass of bacteria in a complex food matrix is not feasible with available technologies, and some method for increasing the concentration of the target bacteria and/or separating the target from the sample matrix is therefore needed prior to detection. Virtually, all current assays require culturing of a sample to allow the organisms initially present to grow and multiply to levels that can be detected effectively. This enrichment of the sample to increase the target concentration requires many hours, and often precludes testing of products that must be processed and sent to consumers while still fresh. Enrichment also presents additional problems for the analyst. Other microorganisms present in the sample also grow and may interfere

Abbreviations: TBS, Tris-buffered saline; BHI, brain heart infusion.

☆ Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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with the growth and/or detection of the target bacteria. The extent of target growth during enrichment can also be strongly dependent on sample history, reflecting injury to the target bacteria by extremes of temperature, pH, etc. This variability in the extent of target growth during enrichment prevents reliable quantitation, and can produce false negative results from samples containing injured bacteria. Rapid assay methods that avoid or minimize enrichment are therefore needed to assure effective microbial quality control for fresh products. Such rapid methods would also be very useful in other food processing and biomedical applications.

If enrichment is not used to raise the target bacteria concentration to detectable levels, then a physical method for isolating bacteria from a large sample volume and concentrating them into a small analysis volume must be employed prior to detection. The concentration factor, reflecting the net increase in bacteria concentration at the detector is given by the product of capture efficiency (the number of bacteria isolated divided by the number present in the sample) and volume ratio (the ratio of sample volume to analysis volume). High concentration factors can be achieved very simply by filtration through sub-micron pore size membrane filters. With samples that are free of particulates, bacteria can be isolated very quickly from large volumes of liquid sample with virtually 100% yield. Protocols have been developed for pretreatment of food samples to permit filtration capture (Tortorello and Stewart, 1994; Yamaguchi et al., 1994), but clogging of the filter generally limits the volume of sample which can be processed. Filtration is non-selective, and detection of the target bacteria can be very challenging if the sample contains other microorganisms at relatively high levels.

These problems can be largely overcome with affinity methods. Immunomagnetic beads have been used for affinity isolation of bacteria in numerous studies (Okrend et al., 1991; Fratamico et al., 1992; Gehring et al., 1996). Typical applications utilize polystyrene spheres approximately 3 μm in diameter with a ferromagnetic core and a surface coating of covalently attached antibody. These beads are combined with ~1 ml sample in a test tube at a concentration of ~10^7/ml, mixed for ~30 min, and separated from the sample using a permanent magnet held against the side of the tube. The beads and captured bacteria can be recovered in a volume as small as 1 μl, providing a concentration factor as high as 1000 for a 1-ml sample. However, immunomagnetic capture is limited to relatively small sample volumes. The concentration of beads must be held near 10^7/ml to achieve efficient recovery (Irwin et al., 2002), and the cost of sufficient beads to treat large sample volumes is considerable. Large numbers of beads can also interfere with subsequent detection of the captured bacteria, either by physically blocking detection, or by adsorbing detection reagents and producing high background signals (Gehring et al., 1996). Other affinity isolation approaches include cloth coated with the cyclic peptide polymyxin (Blais and Yamakazi, 1991). By mixing food samples with small pieces of coated cloth, Gram-negative bacteria were captured via interaction of their negatively charged surface with the multiple positive charges on polymyxin. This approach provided better selectivity than filtration and was not subject to clogging, but was limited to small sample volumes.

The most frequently used format for affinity isolation of small molecules is a column packed with beads, but few publications have appeared on this approach to bacteria isolation. The feasibility of immunoaffinity capture of microorganisms using a column format was first demonstrated (Mattiason, 1983) with a model system consisting of yeast and a column packed with Sepharose beads coated with conconavalin A. Subsequently, an immunoaffinity column was developed for capture of pathogenic bacteria (Molloy et al., 1995). The focus of this work was the removal of bacteria from milk to simulate remediation of heavily contaminated foods, and high bacteria concentrations and large column volumes were used. Capture and detection of low levels of bacteria in sample volumes up to 4 liters has been reported recently (Weimer et al., 2001) using a large volume (~5 ml) bed of 3 mm diameter antibody-coated beads. The use of large support beads and a fluidized bed prevented clogging by sample particulates, but capture efficiency was low and the column volume was not well matched to typical analysis volumes. These studies have clearly shown that bacteria can be captured and concentrated on immunoaffinity columns with volumes of 1–5 ml. However, much smaller columns must be used to achieve high concentration factors and reasonable cost. Optimum

column volume depends on the specific assay parameters, but general trends can be illustrated by considering representative values for sample volume (10 ml), analysis volume (10 μl), detection limit (10^2 bacteria), and immunoaffinity support cost (US$100/ml). A column volume of 1 ml would yield a concentration factor of 10, a detection limit of 1000 bacteria/ml in the raw sample (only 1% of the captured bacteria would be analyzed), and a cost of US$100. A column volume of 10 μl would yield a concentration factor of 1000 with a detection limit of 10 bacteria/ml at a cost of US$1. This report describes the development and characterization of microliter-volume immunoaffinity columns expressly designed for efficient capture and concentration of bacteria from dilute samples. Quantitative results are reported on the effect of bead composition, column volume, and flow rate on capture efficiency of *Salmonellae*.

2. Materials and methods

2.1. Materials

Water was deionized in-house with a Nanopure water treatment system (Barnstead, Dubuque, IA). Tris(hydroxymethyl)aminomethane (Tris)-buffered saline (TBS) was from Pierce (Rockford, IL). Triton X-100 and Tween-20 were from Sigma (St. Louis, MO, USA). Nunclon™ flat-bottomed sterile polystyrene microwell plates with covers were from Fisher Scientific (Philadelphia, PA, USA). Brain heart infusion (BHI) media was from Difco (Detroit, MI, USA). Glass capillary pipettes were from Drummond Scientific (Broomall, PA, USA). Affi-Gel Hz, Affi-Prep Hz, Affi-Gel Hz 10 × coupling buffer, and Affi-Gel Hz oxidizer were from Bio-Rad Laboratories (Hercules, CA). Affinity purified goat antibody to *Salmonellae* Common Structural Antigens and was from Kirkegaard and Perry Laboratories (Gaithersburg, MD). All other chemicals used were of reagent grade. Solutions were filter-sterilized with 0.2 μm membranes.

2.2. Bacteria

Salmonella enteriditis (laboratory isolate obtained from Dr. K. Rajkowski, USDA) and *E. coli* O157:H7 (ATCC 43895) in brain heart infusion broth (BHI, Difco) were grown to stationary phase in shaken Erlenmeyer flasks at 37°. Cells were diluted in TBS to approximately 10^7/ml and the actual cell concentration determined using a Petroff-Hauser counting chamber with phase contrast microscopy. Cells were then diluted in TBS to the desired concentration.

2.3. Apparatus

For bacteria enumeration, an EL 311s Microplate reader (Bio-Tek Instruments, Winooski, VT, USA) equipped with a shaker and temperature control unit was interfaced to a Macintosh Plus computer (Apple Computer, Cupertino, CA, USA) running FreeTerm serial communication software (William Bond, author). The reader measured the absorbance of each well at 450 nm at regular intervals (10–30 min) and transmitted this ASCII text data to the computer. The resulting text file was imported into Excel (Microsoft, Bellingham, WA, USA) and processed using locally written macro procedures to determine the time at which the absorbance in each well reached a specified threshold. For preparation of columns and flow studies, single channel and four channel peristaltic pumps (EconoPump, Bio-Rad Laboratories, Hercules, CA) were used. Pump tubing was sterilized by flowing ethanol for 2 min followed by a TBS rinse.

2.4. Bacteria enumeration

Bacteria were enumerated using a micro-growth assay procedure described elsewhere (Brewster, 2003). Briefly, liquid samples (10–100 μl) were pipetted into individual microplate wells, and BHI media was added to bring the total volume to 200 μl. Standards containing known concentrations of bacteria in TBS were included for calibration. The plate was covered, placed in a temperature-controlled plate reader, and the absorbance of each well was monitored at 450 nm. The time T required for the absorbance to reach the half-maximum was determined, and a plot of T vs. the logarithm of bacteria concentration was used to generate a calibration curve from the standards. Typical standard deviation in T was 0.07 h, giving an uncertainty in concentration of approximately 0.05 log unit. Growth conditions were as follows: wavelength: 450 nm, mode: repeated read, read interval:

900 s, shaking speed: low, shaking duration: 120 s, and temperature: 37 °C.

2.5. Anti-Salmonellae immunoaffinity beads

Two 500 μl aliquots of Affi-Prep Hz or Affi-Gel Hz slurry were washed three times with 1 ml of Affi-Gel Hz 1 × coupling buffer and the supernatant removed. Lyophilized goat anti-Salmonellae antibody (1 mg) was reconstituted in 1 ml coupling buffer, and 20 μl of Affi-Gel oxidizer (periodate) solution was added. The mixture was incubated 30 min in the dark to allow formation of aldehyde groups from carbohydrate moieties on the antibody, then 100 μl of glycerol was added to stop the reaction. Two 600 μl aliquots of the reaction mixture were placed in centrifugal ultrafiltration units and centrifuged at 6000 × *g* for 30 min. The filtrate was discarded, 500 μl of coupling buffer was added to the retained antibody, and the centrifugation was repeated. The purified antibody (free of glycerol and oxidizer) was mixed with 500 μl of coupling buffer and added to 500 μl of washed Affi-Prep. A small amount of the supernatant was withdrawn for protein analysis. The mixture was incubated overnight at room temperature with slow tumbling. After withdrawing a small amount of supernatant for protein analysis, the remaining liquid was discarded and sodium azide added to give a final concentration of 0.02%. Protein analysis by a dye-binding assay indicated that approximately 70% of the antibody bound to the beads (data not shown). In some cases, the slurry was washed three times with TBS to remove unbound antibody, but this did not affect performance of the beads. The slurry was stored at 4 °C until used.

2.6. Immunoaffinity column capture

A small amount of polypropylene wool was placed in a glass column (a 50 μl micropipette, 1.2 × 92 mm) to form a ~ 2-mm long plug. The column and plug were rinsed with ethanol and air-dried. Using a small syringe, immunoaffinity beads were added to the column as a slurry in TBS. Unless otherwise noted, the volume of beads was 20 μl. The column was connected to a peristaltic pump using rubber tubing and rinsed with TBS until all bubbles were removed. Samples containing bacteria in TBS were pumped through the column at the specified flow rate and the eluate solution emerging from the column was collected. Samples and eluates were kept on ice to prevent growth. For recirculation experiments, 5 ml of sample in a 15-ml test tube was continuously pumped through the column. The pump inlet tubing was placed at the bottom of the tube and the outlet from the column was allowed to drop into the test tube. The internal volume of the tubing was approximately 0.8 ml. Samples (100 μl) were withdrawn at 5-min intervals from the test tube. To determine capture efficiency, aliquots (10–50 μl) of the feed and eluate solutions were enumerated using the microgrowth assay. To enumerate bacteria bound to the beads, the column was rinsed with TBS and the flow reversed to flush the immunoaffinity beads into a microfuge tube. The beads were suspended in a known volume of buffer and aliquots were assayed for bound bacteria.

3. Results

3.1. Column capture of bacteria from buffer solutions

Anti-*Salmonellae* Affi-Gel and anti-*Salmonellae* Affi-Prep support materials were evaluated by measuring the capture of bacteria from a suspension of 10^5 *Salmonellae*/ml in TBS pumped at various flow rates through columns containing each support. As shown in Fig. 1, growth curves (optical density vs. time) for the Affi-Prep column eluates shifted to longer times relative to the feed as the flow rate decreased, indicating depletion of the bacteria in the eluates. For the Affi-Gel column, the growth curves were nearly superimposable for all flow rates (data not shown), indicating minimal depletion. Table 1 summarizes the results of this experiment, which indicated a strong dependence of capture efficiency on flow rate.

Column bed volume was studied for three volumes (6, 12, and 20 μl ± 1 μl) and two flow rates (250 and 500 μl/min). One column at each volume was exposed to 2 ml of 10^4 cells/ml *Salmonellae* in TBS at the indicated flow rate. Aliquots (4 × 50 μl) of each column eluant were assayed along with standards at 10^5, 10^4, and 10^3 cells/ml. Results of this study are shown in Table 2 and indicate that capture increased with column volume, although smaller columns could still be useful, particularly at lower flow rates.

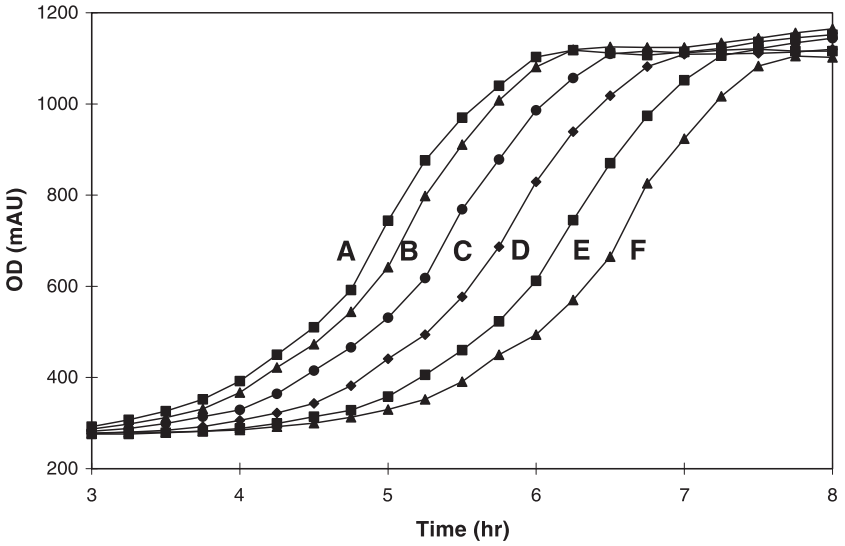

Fig. 1. Growth curves for solutions eluted at various flow rates from anti-*Salmonellae* Affi-Prep column exposed to 10^5/ml *S. enteriditis* in TBS. (A) 10^5 bacteria/ml standard, (B) 800 μl/min, (C) 400 μl/min, (D) 200 μl/min, (E) 100 μl/min and (F) 50 μl/min. Each data point is the mean of four replicates.

A number of controls were incorporated into the experiments to check for non-specific binding, contamination, and other sources of bias or error. Specificity for *Salmonellae* was tested by comparing recovery of *S. enteriditis* and *E. coli* in TBS using anti-*Salmonellae* Affi-Prep columns with 10 μl bed volume at a flow rate of 500 μl/min. The concentration of 10^5 cells/ml *Salmonellae* was depleted by 58% under these conditions, while no depletion of 10^5 cells/ml *E. coli* was detectable (data not shown).

Table 1
Capture efficiency of Affi-Prep and Affi-Gel columns as a function of flow rate for 10^5/ml *Salmonellae* in TBS buffer

Flow (μl/min)	Log[C]	C (cells/ml)	Capture (%)
Affi-Gel			
50	4.90	79507	20
100	4.93	85340	15
200	4.98	94712	5
400	4.95	89589	10
800	4.95	89288	11
Affi-Prep			
50	3.64	4388	96
100	3.95	8862	91
200	4.29	19675	80
400	4.60	39760	60
800	4.83	67624	32

Columns containing only polypropylene wool and polypropylene wool with unmodified Affi-Prep support were included in a number of experiments to test for non-specific binding to the support or the column itself. No detectable non-specific binding was observed in any experiment. Blanks of buffer and media were routinely included to test for contamination. In flow experiments, samples of feed were taken before and after passing through the pump and tubing to test for any change in bacteria concentration due to contamination or retention of bacteria in the tubing. All controls were negative (data not shown).

3.2. Column capture of bacteria from recirculated buffer solutions

A multi-pass approach was also evaluated for isolation of bacteria from larger sample volumes. A 5-ml sample containing bacteria was continuously

Table 2
Recovery of *Salmonellae* as a function of column bed volume

Flow rate (μl/min)	Bed volume		
	6 μl	12 μl	20 μl
250	65%	88%	97%
500	49%	72%	91%

Table 3
Capture efficiency of *Salmonellae* from recirculated buffer solution

Recirculation time (min)	Initial concentration (cells/ml)	
	10^3	10^5
10	33%	42%
20	65%	53%
30	86%	62%
40	89%	87%
50	92%	90%
60	96%	91%

A 5-ml suspension containing bacteria was circulated through a 20-μl bed of anti-*Salmonellae* Affi-Prep beads at 500 μl/min. Samples were taken at 10-min intervals and capture efficiency determined by enumerating the bacteria remaining in the liquid phase. See text for details.

circulated at 500 μl/min through a column containing 20 μl anti-*Salmonellae* Affi-Prep beads. Cumulative capture efficiency was measured as a function of time and initial bacteria concentration. As shown in Table 3, the initial low level of capture increased with time and reached a plateau value greater than 90% after 50 min.

4. Discussion

Two commercial activated affinity supports were evaluated for capture of *Salmonellae*. Affi-Gel is an agarose support with beads 75–300 μm in diameter, suitable for low-pressure filtration conditions. Affi-Prep is a cross-linked acrylamide support with beads 40–60 μm in diameter and is suitable for high pressure, high flow conditions. The hydrazide functional groups on these supports selectively react with carbohydrate side chains of antibodies, covalently linking the antibody to the support without interfering with the antibody-binding site. Several batches of beads were prepared under identical conditions, and protein assays indicated approximately equal antibody loading on both supports. Little or no binding of bacteria to anti-*Salmonellae* Affi-Gel was observed. Anti-*Salmonellae* Affi-Prep bound bacteria effectively and was employed in all further experiments.

For a given sample volume, reducing the column bed volume results in an increased concentration factor and lower detection limit. Low bed volume also reduces cost and potential interference from the beads in subsequent detection steps. Very small bed volumes, on the order of 20 μl, were therefore used with the aim of achieving a concentration factor of 500 with a 10-ml sample. Capture efficiency, determined by measuring depletion of bacteria from the sample following exposure to the beads, was found to be dependent on flow rate and column bed volume, with maximum efficiency occurring for the largest volume and lowest flow rate. Although a flow rate of 500 μl/min only provided ~ 50% capture on a single pass through the column, recirculating the sample allowed over 90% of the bacteria to be captured after five passes.

By concentrating bacteria from a large volume of sample, immunoaffinity isolation can serve the same amplification function as enrichment in assays for low levels of bacteria. The process is much faster than enrichment and also separates the target bacteria from any sample constituents that might interfere with detection. Future research will further optimize parameters such as bead diameter and flow rate, extend this approach to other pathogenic bacteria, and link the isolation step with suitable detection methods to produce rapid, sensitive pathogen assays.

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